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14. ABSTRACT

Prostate cancer is characterized by complex molecular events influenced by diverse genetiand environmental factors. The objective of the present study was to compare the expressiof AMACR and EZH2 in African-American patients and Caucasian patients with prostate canceWe constructed 5 tissue microarrays representing 40 African-American and 159 Caucasian prostate cancer patients and performed immunohistochemistry on these arrays using antibodto AMACR and EZH2. Protein expression was scored on these arrays for both AMACR and EZH2analyzed the data generated from these experiments to investigate the relative levels of two markers in prostate cancer patients from the two racial subgroups and also for any associations with survival patterns and clinico-pathologic parameters. The mean of AMACR expression percentage of PCA patients is significantly higher in Caucasian patientsthan African American patients with prostate cancer. The mean of EZH2 intensity score in CaucaPCA patients is not significantly different from the score in AA PCA patients.

15. SUBJECT TERMS
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Table of Contents

Introduction

Background

With the widespread use of serum prostate-specific antigen (PSA) screening, over 90% of the prostate cancers diagnosed in American men are clinically localized with 100% 5-year survival (*1*). Whether these clinically localized cancers should be treated, and if, treated how aggressively remains an important management dilemma (*2, 3*). However, a significantly higher mortality rate for prostate cancer in Caucasian than in the African-American population (*4*) indicates an inherently aggressive biological nature of the malignancy in the latter. Yet, no conclusive data have emerged to date regarding the natural difference in the biology of prostate cancer in the two racial groups (*5, 6*). It is immensely vital to investigate the biological mechanisms that differentiate indolent prostate cancer from the more aggressive ones in African-American population to discern new pathways that govern the clinical outcome and hold therapeutic potential. Likewise, biomarkers and novel molecular predictors which can identify tumor behavior at the time of diagnosis will be of great clinical utility in tailoring a suitable therapeutic regimen to the predicted aggressiveness of disease.

We reported gene expression profiles of benign prostate, clinically localized prostate cancer, and hormone-refractory metastatic prostate cancer and discovered a cohort of genes which that specifically mark the molecular transition from organ confined prostate cancer to metastatic prostate cancer (*7*). We recently characterized two of these genes, the Polycomb group (PcG) protein Enhancer of Zeste Homolog 2 (EZH2) and AMACR (α-methyacyl-CoA racemase). We demonstrated EZH2 to be overexpressed in metastatic prostate cancer cases and a subset of clinically localized prostate cancer cases associated with poor outcome(*8*). Similarly, we showed AMACR to be overexpressed in prostate cancer using independent experimental methods and prostate cancer specimens(*9*).

AMACR in Prostate Cancer

AMACR plays a key role in peroxisomal ß-oxidation of dietary branched-chain fatty acids and C27-bile acid intermediates(*10*). It catalyzes the conversion of (R)-α -methyl-branched-chain fatty acyl-CoA esters to their (S)-stereoisomers. Only the (S)-stereoisomers can serve as substrates for branched-chain acyl-CoA oxidase during their subsequent peroxisomal ß-oxidation. Two aspects of this pathway may have particular relevance for prostate carcinogenesis: (*a*) the main sources of branched chain fatty acids in humans (milk, beef, and dairy products) , have been implicated as dietary risk factors for prostate cancer (*11*); and (*b*) peroxisomal ß-oxidation generates hydrogen peroxide (*12*) , a potential source of procarcinogenic oxidative damage (*13*).

We reported previously (*9*) the utility of this marker in the detection of prostate adenocarcinoma, via corroboration of increasing expression in immuno-histochemical analysis (IHC) showing an increasing trend in AMACR expression from prostatic intraepithelial neoplasia (PIN) to prostate cancer. Additionally, in this analysis, we quantified the sensitivity and specificity of prostate adenocarcinoma detection via

AMACR staining at 97% and 100% respectively. More recently, our group showed that lower AMACR tissue expression was associated with worse prostate cancer outcome, independent of clinical variables (*14*). This is the first study to show that AMACR expression is significantly associated with prostate cancer progression.

EZH2 in Prostate Cancer

Polycomb group (PcG) proteins are presumed to function in controlling the transcriptional memory of a cell. Miss-expression of PcG proteins can lead to defects in proliferation and tumorigenesis. Our previous studies have shown that dysregulated expression of EZH2 may be involved in the progression of prostate cancer as well as serves as a marker that distinguishes indolent cancer from those at the risk of lethal progression(*8*). We also discovered that EZH2 as a promising biomarker of aggressive breast cancer (*15*), not only extending our initial observations in prostate cancer but also suggesting that EZH2 may have a role in carcinoma progression in malignancies from hormonally regulated tissues.

Our aim in the proposal was (1) *To construct tissue microarray blocks for cohort of African-American men and Caucasian men, for this and future studies* (2) *To delineate if the expression of novel prostate cancer biomarkers, AMACR and EZH2, is different in African-American versus Caucasian men and correlates with clinical outcome* (3) *To determine if the level of immunologic response to AMACR and EZH2 differs in African-American versus Caucasian men and correlates with clinical outcome.*

<u>Research Progress</u>

1. **A. Tissue Microarray design summary**
   B. Overview of distribution of African- American and Caucasian patients in the Tissue Microarrays
   C. Immunohistochemistry and Evaluation for AMACR expression
   D. Immunohistochemistry and Evaluation for EZH2 expression

2. **Statistical Analysis of AMACR for AA and Caucasian PCA Patients**

   - Box plots of AMACR median intensity, median percentage and median product
   - The effect of race and AMACR interaction on clinical outcomes (PCA cases)
   - Survival Analysis of PSA Recurrence: Kaplan-Meier Analysis (PCA cases)
   - Survival Analysis of PSA Recurrence: Cox Models Analysis (PCA cases)

3. **Statistical Analysis of EZH2 for AA and Caucasian PCA Patients**

   - Bar charts of EZH2 median intensity score

- The effect of EZH2 and race interaction associated with clinical parameters (PCA cases)
- Survival Analysis of PSA Recurrence: Kaplan-Meier Analysis (PCA cases)
- Survival Analysis of PSA Recurrence: Cox Models Analysis (PCA cases)

4. **Molecular subtyping of *TMPRSS2* and *Ets* family gene rearrangements in African American and Caucasian patients with prostate cancer using Fluorescent in situ Hybridization**

1. A. Tissue Microarray design summary

For building up tissue microarrays, clinical specimens were obtained from the radical prostatectomy series at the University of Michigan from a cohort of patients who underwent radical retropubic prostatectomy for clinically localized prostate cancer. Consecutive cases were taken to ensure clinical follow-up. Clinical and pathologic data for all patients were acquired with approval from the Institutional Review Board at the University of Michigan. Detailed clinical, pathologic and tissue microarray data are maintained on a secure relational database. Tumors were staged using the TNM system, and graded using the Gleason grading system.

As we reported in the last progress fact sheet, five TMAs are being used for this study. The TMAs were constructed using the manual tissue arrayer (Beecher Instruments, Silver Spring, MD) following a previously described technique (*16, 17*). A spectrum of tissue tissue types including benign, prostate intraepithelial neoplasia (PIN) and prostate cancer were represented on the array.

1. B. Overview of distribution of African- American and Caucasian patients in the Tissue Microarrays

This table summarizes the distribution of the patients across the fine tissue microarrays being used for this study. Benign, PIN and prostate cancer tissues from 40 unique African- American patients and 159 unique Caucasian patients were represented on the 5 TMAs.

TMA	Total cases	Benign	PIN	PCA cases	AA cases	Caucas-ian cases	Other races	Unknown	Caucas-ian GS3+3	Caucasian GS> 3+3	AA GS3+3	AA GS> 3+3
1	54	52	49	54	18	36	0	0	16	19	5	13
2	36	36	33	36	12	24	0	0	12	12	7	5

3	30	28	27	30	10	20	0	0	5	14	1	9
4	55	0	0	54	0	42	0	12	17	22	0	0
5	43	0	0	42	0	37	0	7	16	19	0	0
	218	116	109	216	40	159	0	19	66	86	13	27

Table1. Representative distribution of tissues from African-American (AA) and Caucasian patients on the 5 tissue microarrays. There is an even distribution of cases with Gleason score (GS) 3+3 and more than 3+3 in both the racial subgroups.

1. C. Immunohistochemistry and Evaluation for AMACR expression

Standard avidin–biotin complex immunohistochemistry was used. Antibody concentration was optimized to obtain the strongest target staining without background staining. Monoclonal antibody P504S (Zeta Corp., Sierra Madre, CA, USA) was utilized. Following paraffin removal and hydration, the slides were treated with 0.1 mol/l citrate at pH 6.0 in a pressure cooker and microwaved (15 min on high for P504S and 10 min on high for p-AMACR) for optimal antigen retrieval before immunostaining. Staining was performed on an autostainer (Dako Cytomation, Carpenteria, CA, USA). Sections were incubated sequentially with the primary antibody (1 : 40 dilution P504S for 2 h at room temperature/1 : 5000 dilution p-AMACR for 30 min at room temperature). The Dako Envision Plus detection system was utilized for P504S localization according to the vendor's protocol. Sections were later washed and treated with diaminobenzidine and hydrogen peroxide for 5 min. Sections were counterstained with hematoxylin. Positive staining of AMACR was identified as cytoplasmic and/or luminal/subluminal granular staining within epithelial cells.

Immunohistochemistry evaluation was carried out with ChromaVision ACIS II version (ChromaVision Medical Systems, Inc., San Juan Capistrano, CA (*18*). The ACIS uses preprogrammed advanced color detection software that measures immunohistochemical stains intensity (range, 0–255) and percentage expression (0–100%). All of the images were reviewed to distinguish the tumor from benign areas. Tissue area of interest was electronically circled on the computer screen, and only those areas were used to measure the percentage of the circled cells that stained positive for AMACR (0–100%). The final data were recorded in a Microsoft Excel data sheet and were used for statistical analysis.

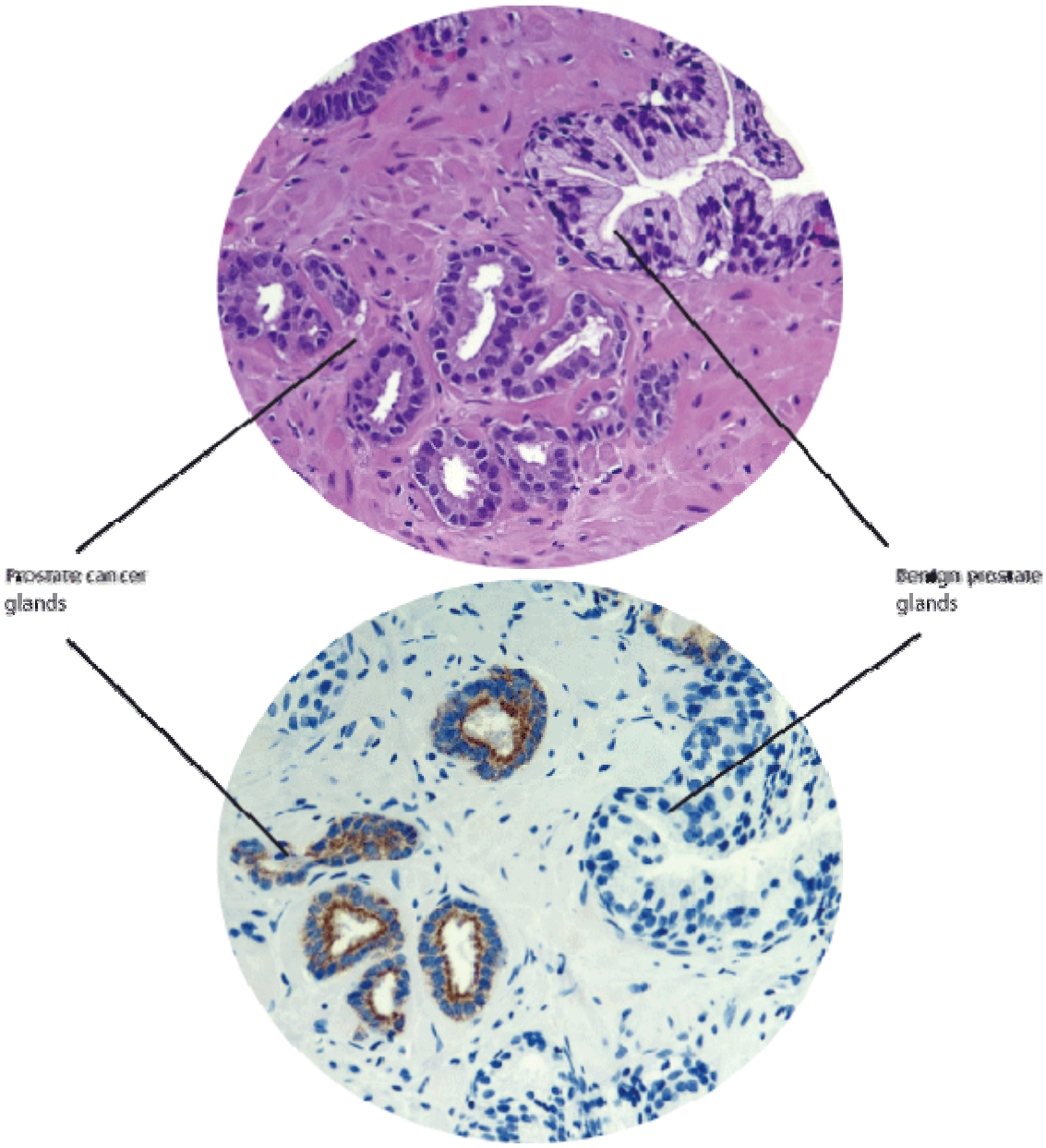


Figure1. Hematoxylin and Eosin staining of benign and prostate cancer glands in the upper image. In the lower image, immunohistochemistry of the same case shows cytoplasmic/ luminal expression of AMACR in the prostate cancer glands preferentially, while the benign glands are negative. Original magnification x100

1. D. Immunohistochemistry and Evaluation for EZH2 expression

Immunohistochemistry was performed on the tissue microarrays by using standard biotin-avidin complex technique and a rabbit polyclonal antibody against EZH2 (Kind gift from. Prof. Otte). Five-µm thick paraffin embedded TMAs were de-waxed and hydrated in

xylene and ethanol respectively. Antigen retrieval was performed in citrate buffer in pressure cooker at pH 6.0 for 15 minutes. The primary antibody was added to the TMA at a 1:100 dilution. The TMA was then treated with a horseradish peroxidase-labeled secondary antibody for 30 minutes, followed by peroxide/diaminobenzidine substrate/ chromagen. The slides were counterstained with hematoxylin. Ezh2 expression was observed in the nucleus, as reported previously (*8*, *19*). Protein expression was scored as negative (score=1), weak (2), moderate (3) and strong (4) in a blinded manner using a validated web- based tool (*20*, *21*).

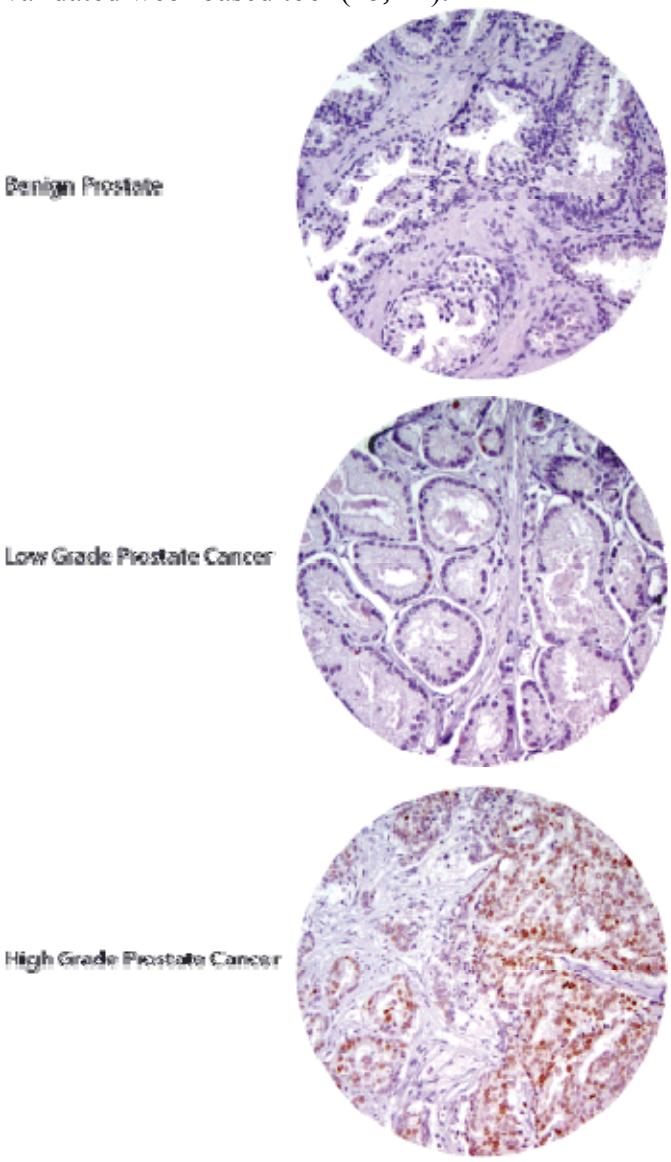


Figure2. Representative tissue elements stained with antibody to EZH2. Immunohistochemical evaluation shows absent nuclear staining in benign prostate, absent or weak staining in a low percent of cells in low grade prostate cancer, and strong expression in high grade prostate cancer. Original magnification x100

2. Analysis of AMACR for AA and Caucasian PCA Patients

1. Box plots of AMACR median intensity, median percentage and median product

Table 2: AMACR dataset race frequency table

Race	Frequency	Percent
African American	40	20.2
Caucasian	159	79.8

Figure 3: AMACR Median intensity
(The median intensity is standardized among each TMA arrays)

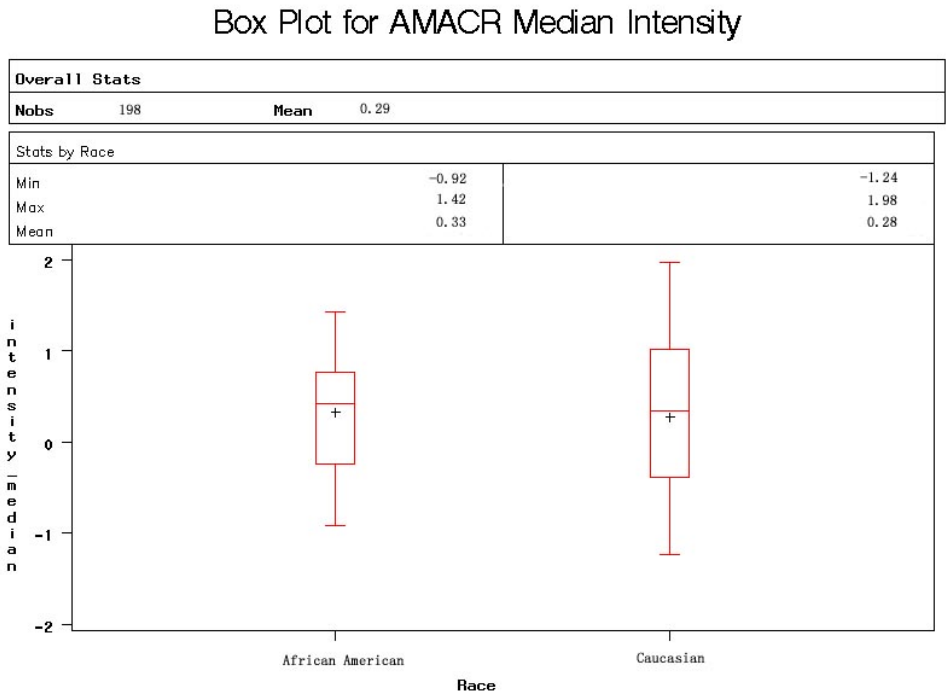


Table 3: Tests of AMACR median intensity comparing two races

P-value	AA vs. Caucasian Mean Intensity
Two smaple student t-test	0.66
Wilcoxon rank sum test	0.72

Figure 4: AMACR Median percentage

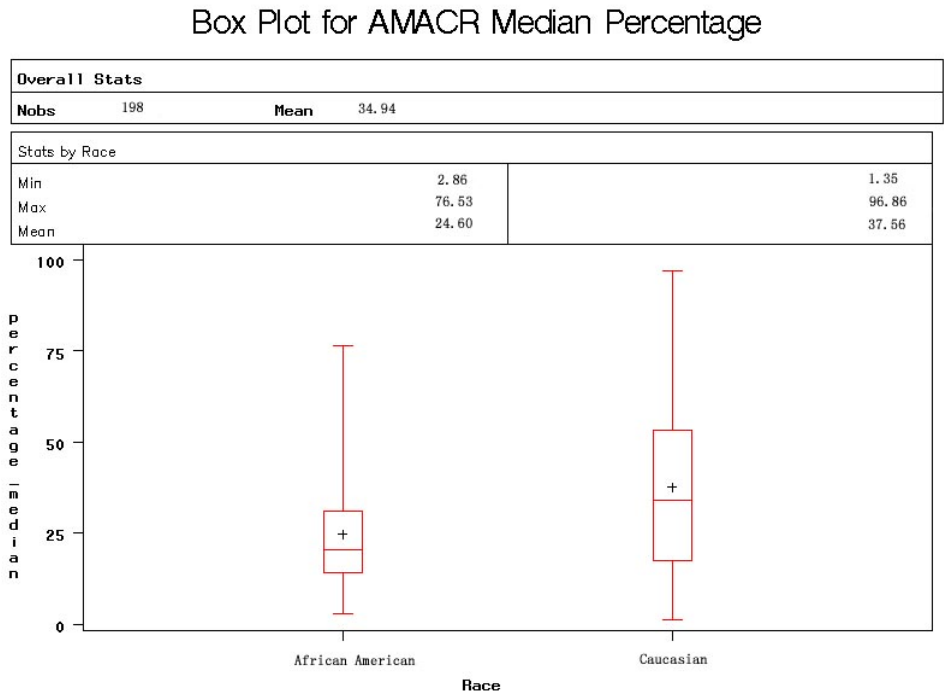


Table 4: Tests of AMACR median percentage comparing two races

P-value	AA vs. Caucasian Mean Percentage
Two smaple student t-test	**<0.0001**
Wilcoxon rank sum test	**0.002**

Of 198 observations, there were 40 African American and 158 Caucasian PCA patients. From three boxplots, the variances of Caucasian are wider than those of AA for the two measurements (intensity, percentage). It shows that the Caucasian PCA patients have a larger variation than AA PCA patients in AMACR expression level. For AMACR median intensity, the two sided student t-test and Wilcoxon rank sum test gives P-values larger than 0.05, which indicates that the difference between mean of Caucasian and AA is not significant in AMACR median intensity. For AMACR median percentage, the P-values from the two tests are less than 0.05, leading to the conclusion that the AMACR median percentage is significantly different between AA and Caucasian. Combined with the box plot, we can see that Caucasian PCA patients have a significantly higher mean of median percentage than AA PCA patients.

2. The effect of race and AMACR interaction on clinical outcomes (PCA cases)

Table 5: Tests of the association between the clinical outcomes and AMACR median intensity or median percentage **among AA patients**

Variable	Median Intensity		Median Percentage	
	Estimate	P-value	Estimate	P-value
Age	-0.56	0.78	-0.01	0.87
Mulitfocal	0.66	0.3	-0.004	0.88
Gland Weight	-0.11	0.1	-0.006	0.036
Gleason Sum	0.12	0.83	0.003	0.91
Tumor Size	0.49	0.006	0.009	0.22
EPE	-0.21	0.78	-0.002	0.94
Surgical Margin	-0.35	0.55	-0.01	0.62
SVI	0.15	0.88	-0.008	0.85
N stage	1.21	0.39	8.00E-04	0.99
Path1997	0.45	0.43	0.006	0.77
basepsa	0.08	0.72	-0.006	0.51

Table 6: Tests of the association between the clinical parameters and AMACR median intensity or median percentage **among Caucasian patients**

Variable	Median Intensity		Median Percentage	
	Estimate	P-value	Estimate	P-value
Age	-0.74	0.3	0.0005	0.98
Mulitfocal	0.08	0.7	0.001	0.86
Gland Weight	-0.03	0.34	9.00E-04	0.42
Gleason Sum	0.33	0.1	0.009	0.18
Tumor Size	0.1	0.14	0.005	0.048
EPE	0.16	0.48	4.00E-04	0.96
Surgical Margin	0.12	0.6	0.003	0.71
SVI	-0.48	0.41	-0.03	0.2
N stage	0.72	0.36	-0.008	0.74
Path1997	0.03	0.89	-0.003	0.67
basepsa	0.05	0.53	0.005	0.04

Table 7: Tests of the association between the clinical parameters and the interaction of race and AMACR median intensity or median percentage

	Median Intensity & Race		Median Percentage & Race	
Variable	Estimate	P-value	Estimate	P-value
Age	-0.013	0.93	0.17	0.87
Mulitfocal	0.29	0.39	-0.002	0.85
Gland Weight	0.08	0.36	0.005	0.17
Gleason Sum	-0.1	0.72	-0.003	0.78
Tumor Size	0.39	0.037	0.007	0.53
EPE	-0.18	0.63	-0.001	0.95
Surgical Margin	-0.23	0.45	-0.007	0.56
SVI	0.31	0.59	0.01	0.62
N stage	0.25	0.76	0.005	0.86
Path1997	0.17	0.54	0.004	0.72
basepsa	-0.04	0.87	0.01	0.19

(Listed are the p-values for the parameter of the interaction term)

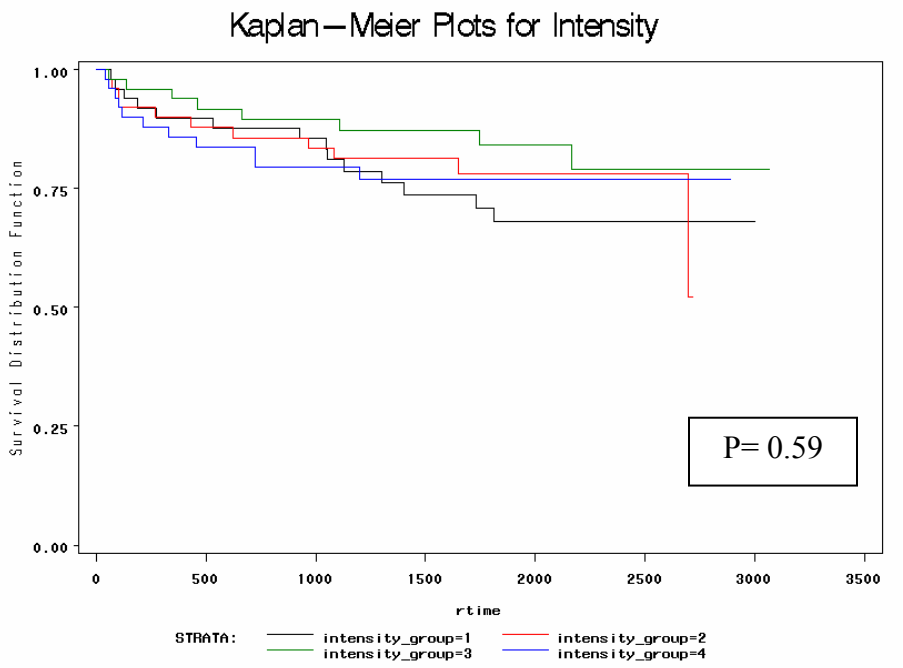
3. Survival Analysis of PSA Recurrence: Kaplan-Meier Analysis (PCA cases)

Table 8: PSA recurrence frequency table

PSA Recurrence		AA	Caucasian	Total
0	Frequency	35	119	154
	Percentage	17.68	60.1	77.78
1	Frequency	5	39	44
	Percentage	2.53	19.7	22.22

(a). Comparing four intensity/percentage groups overall (log-rank test P-value shown in the figure)

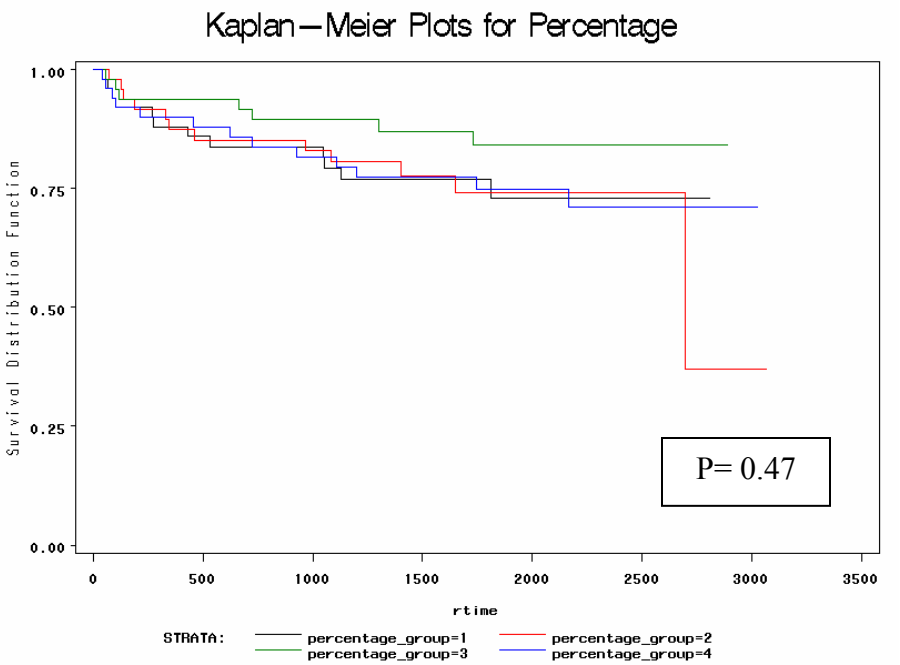
Figure 5: KM plot of AMACR median intensity groups overall



Intensity Group 1:	Median Intensity $\leq$ -0.30
Intensity Group 2:	-0.30$\leq$ Median Intensity $\leq$ 0.38
Intensity Group 3:	0.38 $\leq$ Median Intensity $\leq$ 0.88
Intensity Group 4:	Median Intensity $\geq$ 0.88

(Note: the median intensity is standardized among each TMA arrays)

Figure 6: KM plot of AMACR median percentage groups overall



Percentage Group 1:	Median Percentage ≤ 17.12
Percentage Group 2:	17.12≤ Median Percentage ≤ 28.50
Percentage Group 3:	28.50 ≤ Median Percentage ≤ 48.65
Percentage Group 4:	Median Percentage ≥ 48.65

Table 9: Log rank and Wilcoxon tests overall

	Median Intensity	Median Percentage
DF	3	3
Log rank χ^2	1.93	2.53
P-value	0.59	0.47
Wilcoxon χ^2	1.94	2.21
P-value	0.58	0.53

(b). Comparing four intensity/percentage/product groups stratified on RACE (log-rank test P-value shown in the figure)

Figure 7: KM plot of AMACR median intensity groups stratified on race (Caucasian patients, P= 0.62; African American patients, P= 0.54)

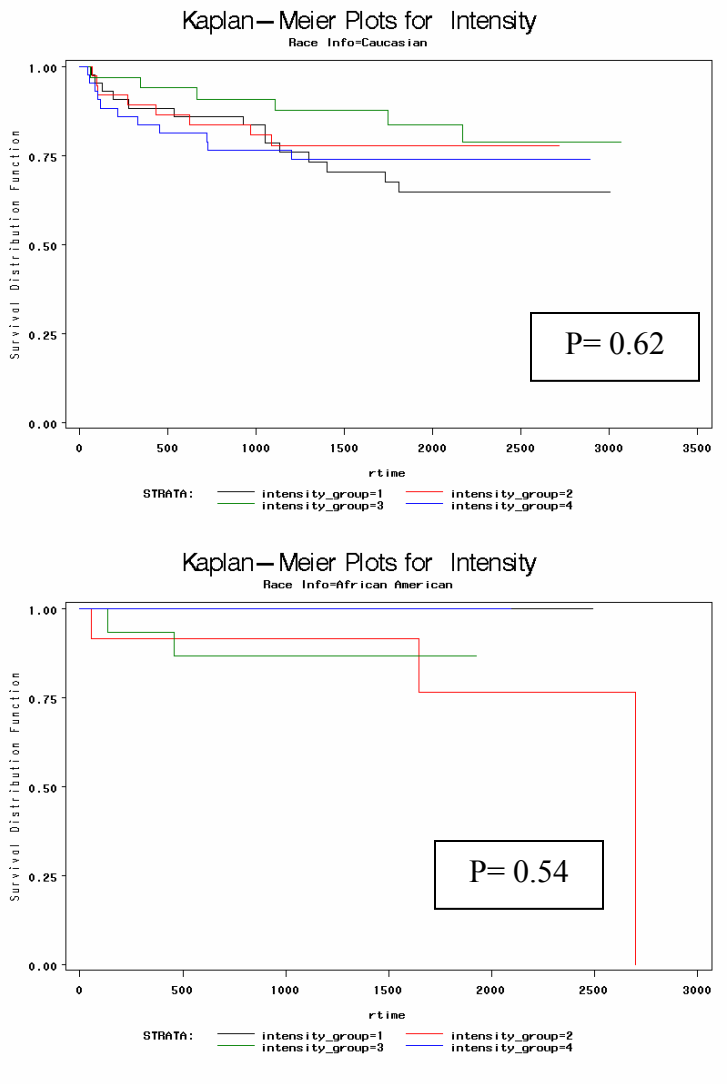


Figure 8: KM plot of AMACR median percentage groups stratified on race (African American patients, P= 0.58; Caucasian patients, P= 0.52)

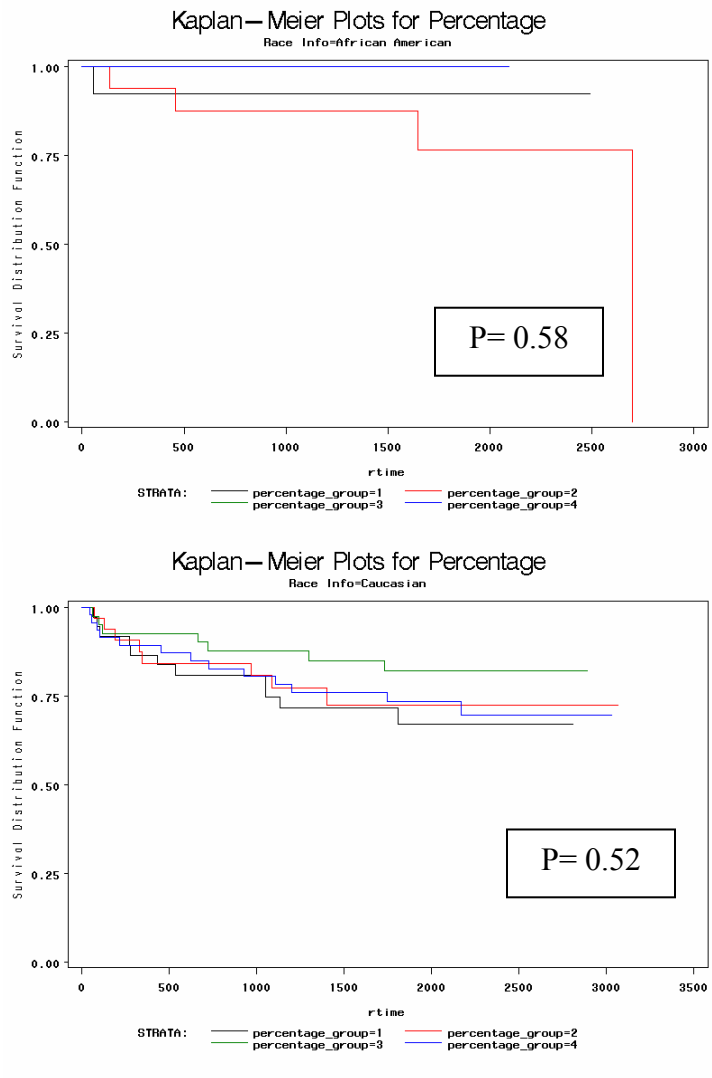


Table 10: Log rank and Wilcoxon tests stratified on race

Measurements	Median Intensity		Median Percentage	
Race	AA	Caucasian	AA	Caucasian
DF	3	3	3	3
Log rank χ^2	1.78	2.16	1.96	2.28
P-value	0.62	0.54	0.58	0.52
Wilcoxon χ^2	1.64	2.30	1.60	2.16
P-value	0.65	0.51	0.66	0.540

It appears from the above plots that there is no obvious trend showing that AMACR expression level is associated with PSA recurrence even when we stratified on race. This is further confirmed by the log-rank tests and Wilcoxon tests (when we focus more heavily on the early follow up).

4. Survival Analysis of PSA Recurrence: Cox Models Analysis (PCA cases)

Table 11: Cox Univariate Model overall

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
Intensity	0.84	0.57	1.23	0.37
Percentage	1	0.99	1.01	0.98
Race	1.17	0.93	1.48	0.18
Age	1.02	0.98	1.06	0.26
Gland Weight	1.00	0.98	1.01	0.81
Gleason Sum	**1.94**	**1.39**	**2.72**	**0.0001**
Size	**2.07**	**1.40**	**3.08**	**0.0003**
Mulitfocal	1.19	0.61	2.32	0.60
EPE	**2.98**	**2.09**	**4.26**	**<0.0001**
SM	**3.63**	**2.26**	**5.83**	**<0.0001**
SVI	**4.12**	**2.54**	**6.69**	**<0.0001**
Path1997	**3.35**	**2.28**	**4.92**	**<0.0001**
Nstage	**5.93**	**1.80**	**19.49**	**0.003**
PSA	**1.03**	**1.02**	**1.05**	**<0.0001**

From the Cox univariate model, we find that risk of PSA recurrence is increased with increasing Gleason Sum (p=0.0001), with increasing size (p=0.0003), with increasing EPE (p<0.0001), with increasing surgical margin [SM] (p<0.0001), with increasing SVI (p<0.0001) and with increasing path 1997 (p<0.0001), with increasing N stage (p=0.0034), with increasing preop-PSA (p<0.0001). The risk of PSA recurrence is also increased with the decreasing of AMACR intensity (hazard ratio is 0.84), however, this effect is not significant (p=0.37).

Table 12: Cox Univariate Model **among AA patients**

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
Intensity	0.94	0.19	4.80	0.95
Percentage	0.98	0.91	1.06	0.60
Age	1.00	0.88	1.15	0.94
Gland Weight	1.00	0.86	1.06	0.34
Gleason Sum	1.52	0.41	5.55	0.53
Size	**4.73**	**1.08**	**20.76**	**0.04**
Mulitfocal	.	.	.	.
EPE	**5.32**	**1.74**	**16.22**	**0.0033**
SM	**4.63**	**1.25**	**17.22**	**0.022**
SVI	**12.48**	**2.72**	**57.35**	**0.0012**
Path1997	**6.28**	**1.85**	**21.28**	**0.0032**
Nstage	**46.39**	**4.10**	**524.88**	**0.0019**
PSA	**1.16**	**1.06**	**1.26**	**0.0017**

Table 13: Cox Univariate Model **among Caucasian patients**

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
Intensity	0.86	0.58	1.27	0.46
Percentage	1.00	0.99	1.01	0.88
Age	1.02	0.97	1.06	0.43
Gland Weight	1.00	0.98	1.01	0.90
Gleason Sum	**2.03**	**1.43**	**2.86**	**<0.0001**
Size	**1.91**	**1.25**	**2.90**	**0.0026**
Mulitfocal	1.13	0.57	2.22	0.74
EPE	**2.65**	**1.80**	**3.89**	**<0.0001**
SM	**3.69**	**2.13**	**6.39**	**<0.0001**
SVI	**3.51**	**1.97**	**6.23**	**<0.0001**
Path1997	**3.05**	**1.96**	**4.74**	**<0.0001**
Nstage	2.52	0.34	18.47	0.3624
PSA	**1.03**	**1.00**	**1.04**	**0.0034**

The Cox univariate model for AA patients is not very powerful, since there are only five events among the whole 40 AA patients.

Table 14: Cox Multivariate Model Overall

Cox Multivariate Model (Intensity)

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
Race	2.78	0.93	8.30	0.07
Intensity	0.43	0.06	3.27	0.41
Race & Intensity	1.92	0.24	15.26	0.54
Gleason	1.42	0.94	2.16	0.1
Age	1.03	0.99	1.08	0.18
Gland weight	0.99	0.98	1.01	0.48
Size	1.36	0.79	2.35	0.27
Mulitfocal	0.52	0.25	1.12	0.09
EPE	1.16	0.50	2.70	0.74
SM	**2.84**	**1.15**	**5.48**	**0.002**
SVI	0.70	0.28	1.76	0.45
Path1997	1.92	0.71	5.23	0.20
Nstage	5.14	0.88	29.84	0.07
PSA	1.02	0.99	1.05	0.15

Cox Multivariate Model (Percentage)

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
Race	1.50	0.11	20.21	0.76
Percentage	0.96	0.85	1.08	0.53
Race & Percentage	1.03	0.92	1.17	0.58
Gleason	1.39	0.92	2.11	0.12
Age	1.03	0.99	1.08	0.17
Gland weight	0.99	0.98	1.01	0.48
Size	1.38	0.79	2.38	0.26
Mulitfocal	**0.51**	**0.24**	**1.10**	**0.08**
EPE	1.23	0.54	2.83	0.62
SM	**2.85**	**1.48**	**5.48**	**0.002**
SVI	0.78	0.34	1.84	0.58
Path1997	1.79	0.68	4.73	0.24
Nstage	3.79	0.74	19.50	0.11
PSA	1.02	0.99	1.05	0.18

Table 15: Cox Multivariate Model **among Caucasian patients**

Cox Multivariate Model (Intensity)

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
Intensity	0.81	0.52	1.27	0.36
Gleason	1.59	1.01	2.51	0.04
Age	1.03	0.98	1.08	0.22
Gland weight	0.99	0.97	1.01	0.56
Size	1.36	0.78	2.39	0.28
Mulitfocal	0.47	0.22	1.02	0.06
EPE	1.26	0.53	3.01	0.6
SM	**2.8**	**1.39**	**5.65**	**0.004**
SVI	0.6	0.23	1.59	0.31
Path1997	1.7	0.62	4.74	0.3
Nstage	5.98	0.63	56	0.12
PSA	1.02	0.99	1.05	0.15

Cox Multivariate Model (Percentage)

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
Percentage	1	0.98	1.01	0.65
Gleason	1.54	0.99	2.41	0.06
Age	1.03	0.98	1.09	0.19
Gland weight	0.99	0.98	1.01	0.55
Size	1.38	0.79	2.44	0.26
Mulitfocal	0.46	0.21	1.01	0.05
EPE	1.29	0.54	3.07	0.56
SM	**2.84**	**1.41**	**5.72**	**0.0035**
SVI	0.7	0.28	1.72	0.43
Path1997	1.63	0.6	4.39	0.33
Nstage	5.43	0.57	51.49	0.15
PSA	1.02	0.99	1.05	0.19

When all clinical variables are considered jointly, only surgical margin remains a significant predictor of increased risk of PSA recurrence when either the whole sample or Caucasian patients considered separately. By using the backward selection, only surgical margin and path1997 are selected as the most parsimonious model in all the Cox multivariate models. However, it is not possible to fit the Cox multivariate model for AA patients. It is because of the small number of events in AA patients (only 5 events in 40 AA patients).

3. Analysis of EZH2 for AA and Caucasian PCA Patients

1. Bar charts of EZH2 median intensity score

Table 16: EZH2 dataset race frequency table

Race	Frequency	Percent
African American	18	33.33
Caucasian	36	66.67

Figure 9: EZH2 Median intensity score bar chart

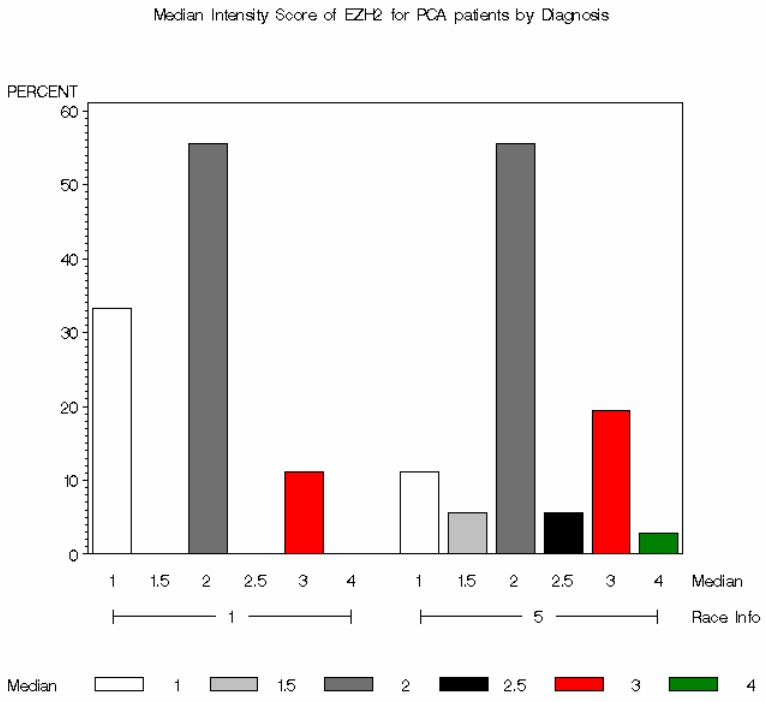


Table 17: Tests of EZH2 median intensity score comparing two races

P-value	AA vs. Caucasian Mean Intensity score
Two sample student t-test	0.063
Wilcoxon rank sum test	0.072

Of 54 observations, there are 18 African American and 36 Caucasian PCA patients. From the bar chart, there are more patients has median intensity score of 2 in both race groups. For Caucasian PCA patients (Race=5), there are more observations having higher median intensity score (≥3) and less having lower intensity score (≤2) than those for AA group. However, the two sample student t-test and Wilcoxon rank sum test give the similar P-value marginally larger than 0.05. It seems that the difference of mean of median intensity score between Caucasian and AA is not significant.

2. The effect of EZH2 and race interaction associated with clinical parameters (PCA cases)

Table 18: Tests of the interaction between the clinical parameters and the interaction of race and EZH2 median intensity score

	EZH2 & Race	
Variable	Estimate	P-value
Age	0.46	0.89
Mulitfocal	0.1	0.85
Gland Weight	-0.02	0.9
Gleason Sum	1.07	0.07
Tumor Size	0.3	0.4
EPE	0.46	0.43
Surgical Margin	0.28	0.57
SVI	0.45	0.66
N stage	0.33	0.76
Path1997	0.67	0.13
basepsa	0.47	0.35

(Listed are p-values for parameter of interaction term: EZH2 Median Intensity Score × Race)

Table 19: Tests of the association between clinical parameters and EZH2 median intensity score **among AA patients**

	EZH2 Intensity Score	
Variable	Estimate	P-value
Age	-0.84	0.76
Mulitfocal	0.65	0.46
Gland Weight	-0.08	0.38
Gleason Sum	1.56	0.13
Tumor Size	0.3	0.36
EPE	1.39	0.18
Surgical Margin	0.98	0.25
SVI	2.56	0.07
N stage	2.42	0.12
Path1997	1.34	0.09
basepsa	0.41	0.31

Table 20: Tests of the association between clinical parameters and EZH2 median intensity score **among Caucasian patients**

	EZH2 Intensity Score	
Variable	Estimate	P-value
Age	-1.3	0.48
Mulitfocal	0.44	0.5
Gland Weight	-0.06	0.44
Gleason Sum	-0.58	0.28
Tumor Size	3.00E-04	0.98
EPE	0.52	0.35
Surgical Margin	0.28	0.64
SVI	1.77	0.22
N stage	1.77	0.22
Path1997	0.32	0.53
basepsa	-0.04	0.88

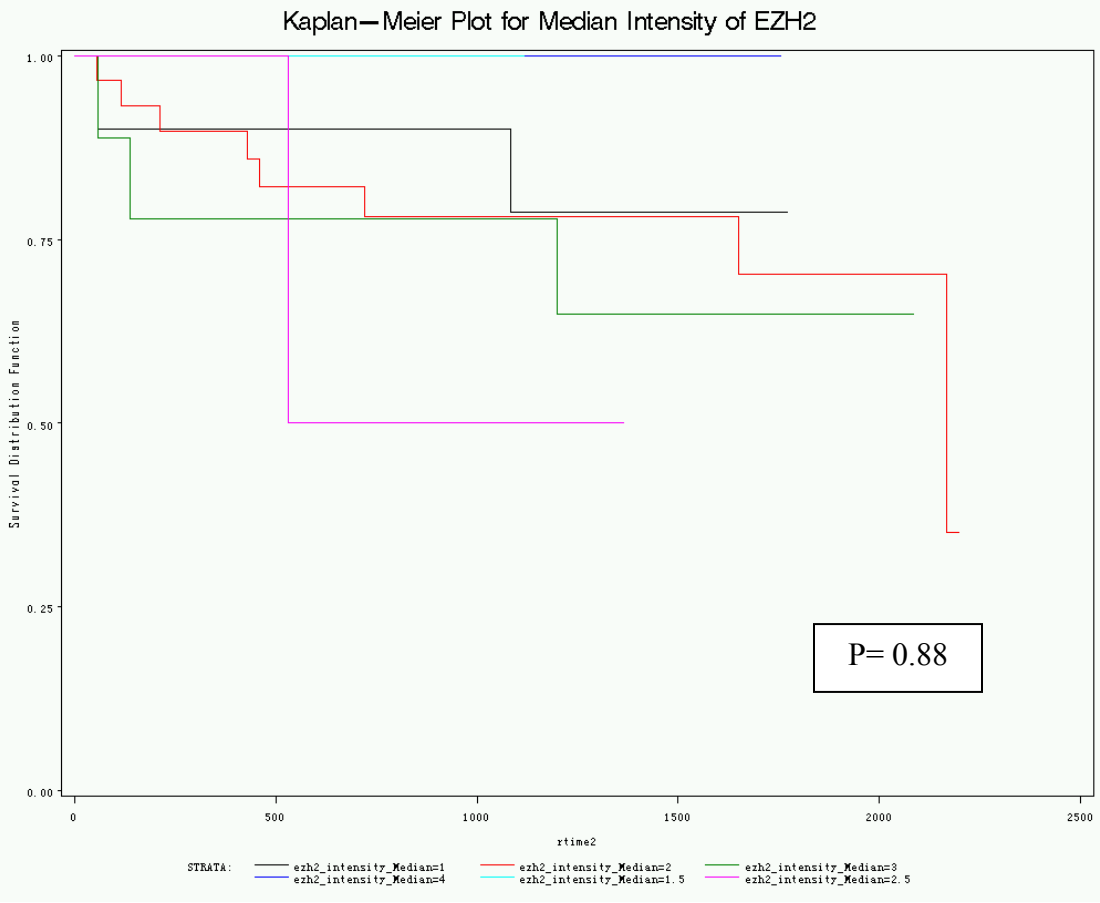
3. Survival Analysis of PSA Recurrence: Kaplan-Meier Analysis (PCA cases)

Table 21: PSA recurrence frequency table

PSA Recurrence		AA	Caucasian	Total
0	Frequency	14	26	40
	Percentage	25.93	48.15	74.07
1	Frequency	4	10	14
	Percentage	7.41	18.52	25.93

(a). Comparing six median intensity score groups overall

Figure 10. KM plot of EZH2 median intensity score groups overall



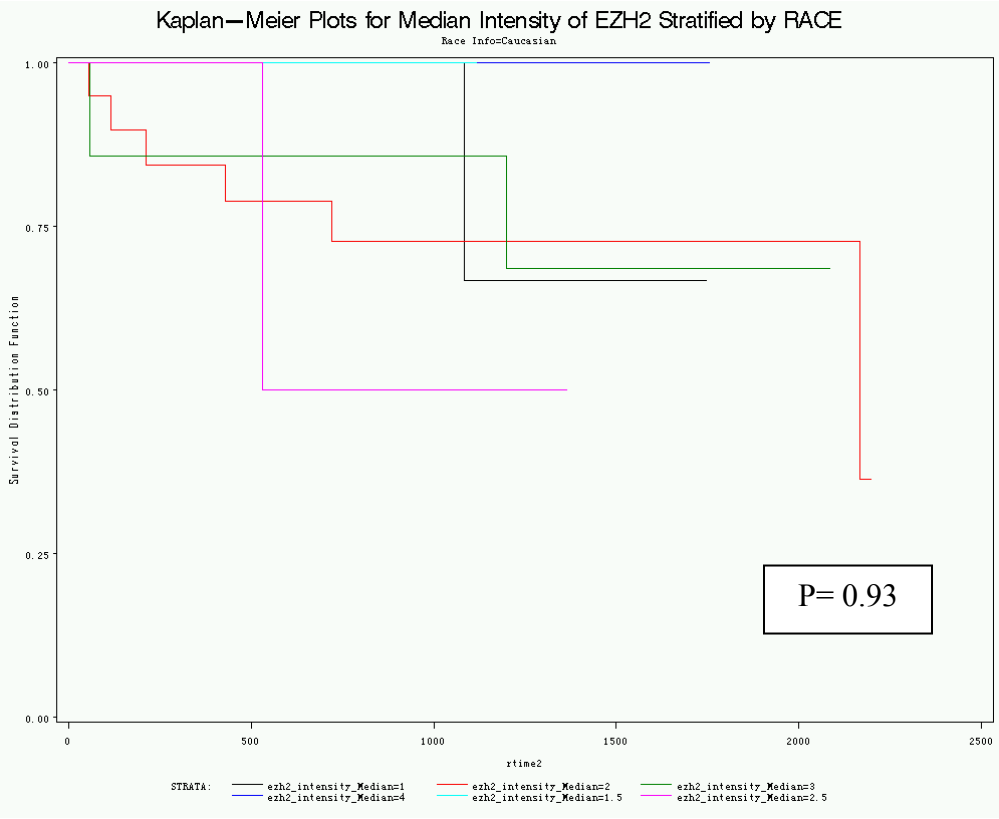
Black	Median Intensity Score Group 1:	Median Intensity Score=1
Red	Median Intensity Score Group 2:	Median Intensity Score=2
Green	Median Intensity Score Group 3:	Median Intensity Score=3
Blue	Median Intensity Score Group 4:	Median Intensity Score=4
Light Blue	Median Intensity Score Group 5:	Median Intensity Score=1.5
Purple	Median Intensity Score Group 6:	Median Intensity Score=2.5

Table 22: Log rank and Wilcoxon tests overall

	Median Intensity Score
DF	5
Log rank χ^2	1.75
P-value	0.88
Wilcoxon χ^2	1.59
P-value	0.90

(b). Comparing six median intensity score groups stratified on RACE (log-rank test P-value shown in the figure)

Figure 11: KM plot of EZH2 median intensity groups stratified on race (Caucasian patients, P= 0.93; African American patients, P= 0.74)



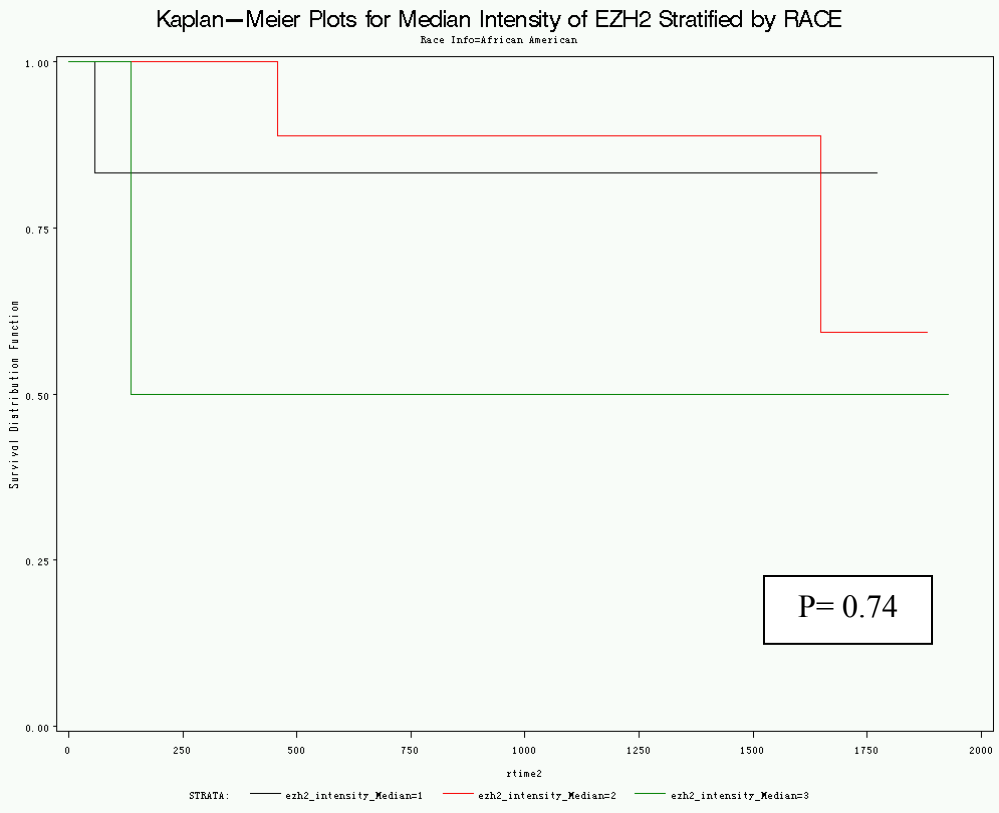


Table 23: Log rank and Wilcoxon tests stratified on race

Measurements	Median Intensity Score	
Race	AA	Caucasian
DF	2	5
Log rank χ^2	0.60	1.32
P-value	0.74	0.93
Wilcoxon χ^2	1.65	1.39
P-value	0.44	0.92

There seems to be some departures between certain intensity score groups. However, the survival curves for intensity score group 4 and 5 remain at value 1 for the whole study. This is due to the small sample size (only 54 observations, 14 events, 40 censored) and there is no event observed for these two groups. The log rank and Wilcoxon tests give the P-value much larger than 0.05 overall. We fail to reject the null hypothesis and there is no significant difference between median intensity score groups. If we stratify on race, we still cannot rule out the null hypothesis by these two tests.

4. Survival Analysis of PSA Recurrence: Cox Models Analysis (PCA cases)

Table 24: Cox Univariate Model

Cox Univariate Model for EZH2 overall

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
EZH2 Intensity Score	1.18	0.55	2.54	0.68
AMACR Intensity	0.99	0.95	1.03	0.68
Race	1.03	0.77	1.39	0.83
Age	1.02	0.95	1.10	0.56
Gland Weight	0.99	0.96	1.03	0.68
Gleason Sum	**5.65**	**1.81**	**17.62**	**0.003**
Size	1.70	0.86	3.34	0.13
Multifocal	1.35	0.37	4.90	0.65
EPE	**3.70**	**1.92**	**7.13**	**<0.0001**
SM	**8.35**	**3.44**	**20.28**	**<0.0001**
SVI	**5.04**	**2.10**	**12.13**	**0.0003**
Path1997	**2.87**	**1.54**	**5.37**	**0.0009**
Nstage	**15.47**	**3.59**	**66.66**	**0.0002**
PSA	**1.07**	**1.03**	**1.11**	**0.0005**

Table 25: Cox Univariate Model **among AA patients**

Cox Univariate Model for EZH2 among AA patients

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
EZH2 Intensity Score	1.55	0.32	7.60	0.59
AMACR Intensity	0.91	0.83	1.00	0.06
Age	1.03	0.90	1.18	0.64
Gland Weight	0.96	0.87	1.07	0.50
Gleason Sum	2.66	0.36	19.41	0.33
Size	2.39	064	8.84	0.19
Multifocal	.	.	.	.
EPE	**3.18**	**1.08**	**9.36**	**0.036**
SM	.	.	.	.
SVI	**6.82**	**1.55**	**30.01**	**0.01**
Path1997	**3.61**	**1.16**	**11.22**	**0.03**
Nstage	**19.10**	**1.69**	**215.39**	**0.02**
PSA	**1.13**	**1.03**	**1.25**	**0.01**

Table 26: Cox Univariate Model **among Caucasian patients**

Cox Univariate Model for EZH2 among Caucasian patients

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
EZH2 Intensity Score	1.00	0.39	2.58	1.00
AMACR Intensity	1.01	0.97	1.06	0.57
Age	1.01	0.92	1.12	0.75
Gland Weight	1.00	0.96	1.03	0.89
Gleason Sum	**12.34**	**2.16**	**70.37**	**0.005**
Size	1.48	0.65	3.36	0.35
Multifocal	0.88	0.22	3.47	0.85
EPE	**5.17**	**1.77**	**15.12**	**0.003**
SM	**7.81**	**2.79**	**21.89**	**<0.0001**
SVI	**34.49**	**2.16**	**551.51**	**0.01**
Path1997	**2.64**	**0.91**	**7.65**	**0.07**
Nstage	**34.49**	**2.16**	**551.51**	**0.01**
PSA	**1.05**	**1.00**	**1.10**	**0.04**

From the Cox univariate model overall, we find that risk of PSA recurrence is increased with increasing Gleason Sum ($p=0.0028$), with increasing EPE ($p<0.0001$), with increasing surgical margin [SM] ($p<0.0001$), with increasing SVI ($p=0.0003$) and with increasing path 1997 ($p=0.0009$), with increasing N stage ($p=0.0002$), with increasing preop-PSA ($p=0.0005$). The risk of PSA recurrence is also increased with the increasing of EZH2 intensity (hazard ratio is 1.18), with the decreasing of AMACR intensity score (hazard ratio is 0.99). However, neither of the effects is significant with both $p=0.68$.

If we stratify on two race groups and fit Cox univariate model again, the result for AA patients is not very powerful, since there are only four events among the whole 28 AA patients. The results of Cox univariate model for Caucasian patients are very similar to the Cox univariate model for the whole dataset.

Table 27: Cox Multivariate Model overall

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
Race	0.05	0	98.11	0.43
EZH2 Intensity Score	0.15	0.003	6.98	0.33
Race & EZH2 Intensity Score	7.16	0.16	319.72	0.31
Gleason Sum	0.89	0.16	5.13	0.90
Age	1.19	0.99	1.43	0.06
Gland weight	**0.95**	**0.90**	**1.00**	**0.043**
Size	0.36	0.08	1.52	0.16
Multifocal	0.16	0.01	2.35	0.18
EPE	13.98	0.81	242.40	0.07
SM	**20.46**	**3.72**	**112.48**	**0.0005**
SVI	0.34	0.002	56.81	0.68
Path1997	0.45	0.03	7.04	0.57
Nstage	0.42	0.02	11.79	0.61
PSA	**1.25**	**1.09**	**1.45**	**0.002**

Table 28: Cox Multivariate Model **among Caucasian patients**

Variable	Hazard Ratio	Lower 95%CI	Upper 95% CI	P-value
EZH2 Intensity Score	0.69	0.04	11.41	0.8
Gleason Sum	6.35	0.39	103.82	0.19
Age	1.01	0.79	1.29	0.95
Gland weight	**0.98**	**0.91**	**1.05**	**0.5**
Size	0.65	0.14	3.05	0.58
Multifocal	0.13	0.005	3.1	0.21
EPE	97.36	2.34	4047.3	0.02
SM	**66.3**	**2.66**	**1652.22**	**0.01**
SVI	0.06	0	1.54.68	0.57
Path1997	0.05	0.001	2.24	0.12
Nstage	.	.	.	.
PSA	**1.25**	**1.04**	**1.49**	**0.01**

When all clinical variables are considered jointly, surgical margin and preop SA level remain as the significant predictors of increased risk of PSA recurrence when either the whole sample or only Caucasian patients are considered. Age and EPE are marginally significant in the model of the whole dataset (p=0.0577, p=0.0699). Gland weight is marginally significant in the model of only Caucasian patients. After backward selection,

EPE, surgical margin, path1997, preop-PSA remains in both of the models. However, this is not a very powerful test because of the few events. It is not possible to fit the Cox multivariate model for AA patients. It is because of the small number of events in AA patients (only 5 events in 40 AA patients).

Summary of statistical analysis of tissue microarray data evaluated for immunohistochemical expression of AMACR and EZH2

1. The mean of AMACR expression percentage of PCA patients is significantly higher in Caucasian than in African American. However, AMACR intensity does not show any statistical significant difference between the two race groups. Survival analysis reveals that the risk of PSA recurrence is increased with the decrease of AMACR intensity, however, this effect is not statistically significant. The interaction of race and AMACR is neither significant predictor for the PSA recurrence.

2. The mean of EZH2 intensity score in Caucasian PCA patients is not significantly different from the score in AA PCA patients. No clinical parameter is associated with the interaction of EZH2 and race. Survival analysis reveals that the risk of PSA recurrence is increased with the increase of EZH2 intensity. However, this effect is not statistically significant. The interaction of race and EZH2 is neither significant predictor for the PSA recurrence.

4. Molecular subtyping of *TMPRSS2* and *Ets* family gene rearrangements in African American and Caucasian patients with prostate cancer using Fluorescent in situ Hybridization

Four μm thick tissue micro array sections were used for interphase FISH, processed and hybridized as described previously(*22*). Slides were examined using an Axioplan ImagingZ1 microscope (Carl Zeiss) and imaged with a CCD (Charged couple device) camera using the ISIS software system in Metafer image analysis system (Meta Systems, Altlussheim, Germany). FISH signals were scored manually (100x oil immersion) in morphologically intact and non-overlapping nuclei and a minimum of 50 cancer cells or the maximum numbers of cancer cells available in three cores from a case were recorded. Cases without 50 evaluable cancer cells were reported as insufficient. Cores with very weak signals or lack of signals were recorded as insufficient for hybridization. Cases lacking tumor tissue in all three cores were also excluded.

All BACs were obtained from the BACPAC Resource Center (Oakland, CA), and probe locations were verified by hybridization to metaphase spreads of normal peripheral lymphocytes. For detection of *TMPRSS2*, *ERG* and *ETV4* rearrangements we used the following probes: RP11-35C4 (5' to *TMPRSS2*) and RP11-120C17 (3' to *TMPRSS2*), RP11-95I21 (5' to *ERG*) and RP11-476D17(3' to *ERG*), and RP11-100E5 (5' to *ETV4*) and RP11-436J4 (3' to *ETV4*). For detection of *TMPSS2-ETV1* fusion, RP11-35C4 (5' to *TMPRSS2*) was used with RP11-124L22(3'to *ETV1*). BAC DNA was isolated using a QIAFilter Maxi Prep kit (Qiagen, Valencia, CA), and probes were synthesized using digoxigenin- or biotin-nick translation mixes (Roche Applied Science, Indianapolis, IN). The digoxigenin and biotin labeled probes were detected using fluorescein conjugated anti-digoxigenin antibodies (Roche Applied Science) and Alexa 594 conjugated streptavidin (Invitrogen, Carlsbad, CA), respectively.

The tissue microarrays will be evaluated for rearrangements for *TMPRSS2* and members of the *Ets* family.

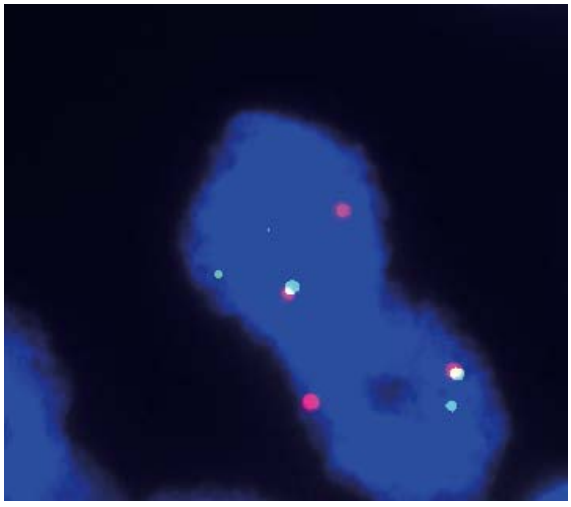


Image of prostate cancer cases showing *ERG* rearrangement

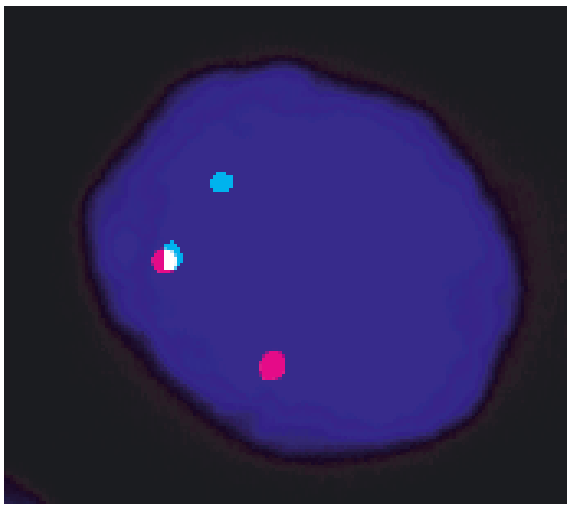


Image of prostate cancer cases showing *TMPRSS2* rearrangement

Future goals

AMACR and EZH2 are promising molecules involved in multiple biological pathways. Earlier it has been shown that their expression is upregulated in prostate cancer. We have analyzed the data generated from the immunohistochemical evaluation of tissue microarrays to compare the expression pattern of the two proteins in Caucasian patients and African- American patients. We analyzed comprehensively the association of these markers with clinico-pathological parameters in African-American patients and Caucasian patients with prostate cancer and also performed univariate and multivariate Cox proportional hazards model of statistically significant covariates to find the link between AMACR and EZH2 expression and prostate cancer recurrence in the two racial groups. *We will do further analysis to investigate the effect of the three way interaction of race, EZH2 and AMACR on clinical outcomes (PCA cases).*

Further, we will look for the differences between the immunologic responses to the two biomarkers in the prostate cancer patients from the two groups. We have recently discovered recurrent gene fusions of the 5' untranslated region of TMPRSS2 to ERG or ETV1 in prostate cancer tissues with outlier expression (*22*). We have performed Fluorescent in situ hybridization on these arrays using *TMPRSS2* and *Ets* family probes to compare the incidence of these gene fusions in the African-American and the Caucasian patients with prostate cancer. We will evaluate any association of these gene fusions with outcome and clinico-pathological parameters in the two racial sub-groups.

Key research accomplishments

1. We have constructed 5 tissue microarrays representing spectrum of prostate pathology including benign glands, prostate intraepithelial neoplasia (PIN) and prostate cancer from 40 African-American and 159 Caucasian patients with prostate cancer.

2. We have stained all these arrays with Hematoxylin and Eosin; and have performed immunohistochemical staining using antibodies to AMACR and EZH2 and have evaluated AMACR and EZH2 protein expression on these tissue microarrays.

3. We have performed biostatistics analysis to compare expression of AMACR and EZH2 in the African-American and Caucasian patients, to look for association with clinico- pathologic parameters, and with outcome in the two racial groups.

4. We have performed Fluorescent in situ hybridization on these arrays with TMPRSS2 and Ets family probes to look for these gene rearrangements in African American and Caucasian patients with prostate cancer.

Reportable Outcomes

1. We have discovered and reported the presence of TMPRSS2 and Ets family gene rearrangements in approximately 70% of American males with prostate cancer (*23*). Comprehensive assessment of TMPRSS2 and ETS family gene aberrations in clinically localized prostate cancer. **Mehra R**, Tomlins SA, Shen R, Nadeem O, Wang L, Wei JT, Pienta KJ, Ghosh D, Rubin MA, Chinnaiyan AM, Shah RB. **Mod Pathology**. 2007 Mar 2

2. We have demonstrated the gene expression profiling status across prostate cancer progression using laser captured cells from a cohort of localized and metastatic prostate cancer patients (*24*). Integrative molecular concept modeling of prostate cancer progression. Tomlins SA, **Mehra R**, Rhodes DR, Cao X, Wang L, Dhanasekaran SM, Kalyana-Sundaram S, Wei JT, Rubin MA, Pienta KJ, Shah RB, Chinnaiyan AM. **Nature Genetics**. 2007 Jan; 39(1):41-51. Epub 2006 Dec 17.

3. We have demonstrated that TMPRSS2:ERG gene fusions can be detected in the urine of patients with prostate cancer; this study supports larger studies on prospective cohorts for noninvasive detection of prostate cancer (*25*). Noninvasive detection of TMPRSS2:ERG fusion transcripts in the urine of men with prostate cancer. Laxman B, Tomlins SA, **Mehra R**, Morris DS, Wang L, Helgeson BE, Shah RB, Rubin MA, Wei JT, Chinnaiyan AM. **Neoplasia**. 2006 Oct;8(10):885-8.

4. We have generated 5 tissue microarrays representing spectrum of prostate pathology, with benign, PIN and prostate cancer samples from 218 patients. These tissue microarrays will be used in the future for detection of proteins by immunohistochemistry; fluorescent in-situ hybridization; RNA in situ hybridization; in situ mi RNA detection, etc.

Conclusion

We have identified a cohort of African- American and Caucasian patients who underwent radical prostatectomy for prostate cancer at the University of Michigan. Five tissue microarrays were constructed from tissues representing 40 African-American and 159 Caucasian patients. AMACR and EZH2 protein expression was explored on these tissue-microarrays using immunohistochemistry. The tissue- microarrays were stained using antibodies to AMACR and EZH2. The tissue microarrays were assessed for the intensity of AMACR and EZH2 expression and the percentage of the cells with that intensity using previously validated methods.

We performed biostatistical analysis on data derived from immunohistochemical evaluation of AMACR and EZH2 on these tissue microarrays. The mean of AMACR expression percentage of PCA patients is significantly higher in Caucasian patients than in African American patients with prostate cancer. However, AMACR intensity does not show any statistical significant difference between the two race groups. The mean of EZH2 intensity score in Caucasian PCA patients is not significantly different from the score in AA PCA patients. The interaction of race and AMACR; or race and EZH2 is neither significant predictor for the PSA recurrence. We will further analyze to look for association of AMACR and EZH2 with clinical parameters in the African American and Caucasian sub-groups. We will look for the immunologic responses to these two markers in the two racial subgroups and also identify molecular subtypes of *TMPRSS2* and *Ets* family members in the two sub-populations.

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Appendices

Abbreviations

AMACR	α- methyacyl-CoA racemase
EZH2	Enhancer of Zeste Homolog 2
IHC	Immunohistochemistry
PCA	Prostate Cancer
PcG	Polycomb group proteins
PIN	Prostatic intraepithelial neoplasia
RT-PCR	Reverse transcriptase polymerase chain reaction
TMA	Tissue Microarray
TNM	Primary tumour (T), Regional lymph nodes (N), Distant metastasis (M)